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## The Clinically Approved Antifungal Drug Posaconazole Inhibits Human Cytomegalovirus Replication

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1     **The Clinically Approved Antifungal Drug Posaconazole Inhibits Human**  
2                                   **Cytomegalovirus Replication**

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11     Running Head: HCMV Inhibition by Posaconazole

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24 **ABSTRACT**

25 Posaconazole (PCZ) is a clinically approved drug used predominantly for prophylaxis and  
26 salvage therapy of fungal infections. Here, we report its previously undescribed anti-human  
27 cytomegalovirus (HCMV) activity. By antiviral assays we demonstrated that PCZ, along  
28 with other azolic antifungals, has a broad anti-HCMV activity being active against different  
29 strains including low-passage clinical isolates and strains resistant to viral DNA polymerase  
30 inhibitors. Using a pharmacological approach, we identified the inhibition of human  
31 cytochrome P450 51 (hCYP51) or lanosterol 14 $\alpha$  demethylase, a cellular target of  
32 posaconazole in infected cells, as a mechanism of anti-HCMV activity of the drug. Indeed,  
33 hCYP51 expression was stimulated upon HCMV infection and the inhibition of its  
34 enzymatic activity by either a lanosterol analog (VFV) or PCZ decreased HCMV yield and  
35 infectivity of released virus particles. Importantly, we observed that the activity of the first  
36 line anti-HCMV drug ganciclovir was tenfold boosted by PCZ and that GCV and PCZ act  
37 synergistically in inhibiting HCMV replication. Taken together, these findings suggest that  
38 this clinically approved drug deserves further investigation in the development of host-  
39 directed antiviral strategies as a candidate anti-HCMV drug with a dual antimicrobial effect.

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41 **KEYWORDS:** HCMV, antiviral, posaconazole, human CYP51, drug repurposing,  
42 synergism.

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47 Human cytomegalovirus (HCMV) is a ubiquitous beta-herpesvirus that infects from 60% to  
48 nearly 80% of the human population worldwide and establishes a life-long persistence in the  
49 host characterized by sporadic reactivations in healthy individuals (1). HCMV is also a major  
50 opportunistic human pathogen, which causes life-threatening diseases in subjects with  
51 acquired or developmental immunodeficiency, such as transplant recipients or immune naïve  
52 fetuses. Indeed, HCMV causes deafness and neurological disorders in approximately 0.1% of  
53 congenital infections (2). Moreover, HCMV has been suggested as a cofactor of vascular  
54 diseases and immune senescence (3).

55 To date, to prevent and treat HCMV infections, there is no vaccine available, and only a  
56 limited number of drugs are licensed for treatment: ganciclovir (GCV), its oral prodrug  
57 valganciclovir, foscarnet, acyclovir, its prodrug valacyclovir, cidofovir, and the recently  
58 approved letermovir (4,5). Currently, all the available anti-HCMV drugs target virus-  
59 encoded proteins, i.e., the DNA polymerase and the terminase (5). The clinical use of these  
60 antivirals can have several drawbacks, such as an unfavorable safety profile characterized by  
61 severe acute or long-term toxicity and poor oral bioavailability (4). Moreover, no drug has  
62 been approved for the treatment of congenital infection (6) and the management of HCMV  
63 infections can be further complicated by the emergence of drug-resistant HCMV strains  
64 (7,8). For all these reasons, there is still a strong need to develop new, safe, and effective  
65 antiviral compounds, possibly endowed with a new mechanism of action.

66 HCMV, like many other viruses, hijacks several cellular pathways and deeply alters many  
67 host physiological processes in order to replicate efficiently (9). Thus, the development of  
68 host-directed antiviral strategies could be an alternative or an additional approach to treat  
69 HCMV-associated diseases (10), as well as to overcome viral resistance issues. In this

70 regard, drug repurposing can represent a powerful strategy for the identification of host  
71 pathways and factors playing a role during viral replication (11-13). Based on this rationale,  
72 we followed this strategy to identify new compounds and targets for anti-HCMV  
73 intervention through a drug screening and follow-up studies that identified several novel  
74 HCMV inhibitors, including both approved drugs and natural compounds (12,14,15).

75 Here, we report the discovery of the previously undescribed anti-HCMV activity of  
76 posaconazole (PCZ), a drug approved for the prophylaxis and salvage therapy of systemic  
77 fungal infections. Our study stems from the observation that drugs belonging to the class of  
78 azolic antifungals were identified in two previous independent drug repurposing screenings  
79 for inhibitors of HCMV replication (12,16,17). Starting from this observation, we initially  
80 evaluated the antiviral activity of a panel of azolic antifungals used both topically and for the  
81 treatment of invasive fungal infections. PCZ, which was not present in our original screened  
82 library, showed an interesting anti-HCMV activity, thus we decided to characterize further  
83 its antiviral mechanism. In the recent years, the antiviral properties of azolic antifungals has  
84 been an emerging topic and PCZ, together with itraconazole, was found to have activity  
85 against both positive-stranded RNA viruses, such as picornaviruses and flaviviruses  
86 (11,18,19), and against negative-stranded RNA viruses, such as Ebola virus and influenza  
87 virus (20,21).

88 We investigated deeper the anti-HCMV activity of PCZ and found that its antiviral  
89 activity was related to its inhibitory activity against human cytochrome P450 51 (hCYP51, or  
90 lanosterol 14 $\alpha$  demethylase). This enzyme is the most conserved cytochrome P450, as well  
91 as the only that catalyzes demethylation of lanosterol (22), being involved in both  
92 cholesterologenesis and synthesis of essential regulatory sterols during meiosis (23). Fungal

93 CYP51 is the target of PCZ and other antifungals in the treatment of mycosis, however our  
94 study suggests that human CYP51 is involved in the antiviral mechanism of PCZ against  
95 HCMV.

96 Noteworthy, our data indicate that a combination of PCZ with GCV, the gold standard for  
97 anti-HCMV therapy, greatly potentiates the antiviral effect of the latter and is synergistic in  
98 inhibiting HCMV replication in infected cells. Thus, this study provides the rationale for a  
99 possible clinical validation of the anti-HCMV activity of PCZ alone or in combination with  
100 GCV.

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## 103 RESULTS

104 **Anti-human cytomegalovirus activity of approved azolic antifungals.** In a drug  
105 repurposing campaign, we previously identified a series of molecules endowed with anti-  
106 HCMV activity, among which we noticed an overrepresentation of drugs belonging to the  
107 class of azolic antifungals (12). To extend this knowledge, a more complete panel of azolic  
108 antifungal drugs was analyzed for anti-HCMV activity (Table 1). As shown in Fig. 1A, in  
109 plaque reduction assays (PRA), posaconazole (PCZ) and ketoconazole (KTZ) showed a  
110 dose-dependent inhibitory effect on HCMV AD169 replication in HFF cells, similarly to  
111 what we previously reported for clotrimazole, econazole, and miconazole (12). On the other  
112 hand, under the same experimental conditions, fluconazole (FCZ), voriconazole (VCZ), and  
113 itraconazole (ITZ) did not exhibit significant anti-HCMV activity (Fig. 1B).

114 Then, to exclude the possibility that the antiviral activity of PCZ might be due to  
115 cytotoxicity, its effect on the viability of uninfected HFFs cells was evaluated by MTT

116 assays. As reported in Table 1, we found that the antiviral activity of posaconazole was not  
117 due to cytotoxicity of the target cells, since a significant toxic effect was not observed at  
118 concentrations up to 250  $\mu$ M.

119 **Broad anti-HCMV activity of posaconazole and other antifungals.** To investigate  
120 the spectrum of anti-HCMV activity of PCZ and of other antifungals, we repeated the  
121 antiviral assays with a panel of HCMV strains, including three different low passage-number  
122 clinical isolates (TB40-UL32-EGFP, VR1814, and 388438U). As reported in Figure 2, the  
123 anti-HCMV activity of PCZ was not dependent on the viral strain, since the  $EC_{50}$  values  
124 obtained with different HCMV strains were comparable (Table 2). Next, we evaluated the  
125 activity of PCZ against HCMV strains resistant to the available viral DNA polymerase  
126 inhibitors, as the emergence of drug resistance is an increasing cause of transplant failure  
127 associated with HCMV infections, in particular after prolonged antiviral therapy (24). PCZ  
128 fully inhibited the replication of viruses with mutations in *UL54* gene conferring cross-  
129 resistance to GCV and cidofovir or to foscarnet and acyclovir (strains 759<sup>T</sup>D100 and  
130 PFA<sup>T</sup>D100, respectively, Table 2). Anti-HCMV activity against different strains was found  
131 also for MCZ and ECZ (Table 2), two other antifungal drugs emerged for anti-HCMV  
132 activity during the drug repurposing screening (12).

133 Finally, the inhibitory activity of PCZ against HCMV resulted not cell type-  
134 dependent, since the  $EC_{50}$  values measured for the TR strain in epithelial ( $EC_{50}$  = 4.2  $\mu$ M)  
135 and endothelial ( $EC_{50}$  = 4.8  $\mu$ M) cells were in line to that observed in fibroblasts ( $EC_{50}$  = 3.7  
136  $\mu$ M) (Table 2). Moreover, since TR strain is naturally resistant to GCV and CDV, we  
137 obtained further evidence of the activity of PCZ against HCMV clinical strains resistant to  
138 DNA polymerase inhibitors.

139 Taken together these results indicated that PCZ and other antifungals could have a  
140 mechanism of action different from that of anti-HCMV drugs targeting the viral DNA  
141 polymerase.

142 **Effects of PCZ treatment on HCMV yield.** PCZ is routinely used as a systemic  
143 drug to prevent and treat fungal infections in immunosuppressed patients (25), thus, we  
144 decided to focus on this molecule, given that a potential dual antifungal and antiviral effect  
145 could be relevant in this clinical setting. To better characterize the anti-HCMV activity of  
146 PCZ, we also evaluated its effects on the production of virus progeny in a multi-cycle viral  
147 growth experiment. PCZ reduced the production of HCMV infectious progeny in a dose-  
148 dependent manner as revealed by virus yield reduction assays (Table 3). In sum, we observed  
149 that treatment of HCMV-infected cells with PCZ after infection resulted in the inhibition of  
150 viral replication and progeny production.

151 **Inhibition of human CYP51, a host target of posaconazole, correlates with anti-**  
152 **HCMV activity.** PCZ is known to interfere with different host pathways (26-29), among  
153 which there are cholesterol homeostasis and synthesis mediated by human CYP51, the rate-  
154 limiting enzyme in late-stage cholesterologenesis (28-30). Thus, we exploited VFV, the most  
155 potent enzymatic inhibitor of hCYP51 identified so far (30), to investigate the involvement  
156 of hCYP51 during HCMV replication. Like other azolic compounds, VFV inhibits human  
157 CYP51 by binding in the enzyme active site and competing with the substrate lanosterol. In  
158 enzymatic assays *in vitro*, both VFV and PCZ inhibited the initial rate of lanosterol  
159 conversion catalyzed by purified hCYP51 with IC<sub>50</sub> values of 0.5 and 8  $\mu$ M, respectively  
160 (Fig. 3A and Table 3). This was not observed with VCZ (IC<sub>50</sub> >100  $\mu$ M), which served as a  
161 negative control in the enzymatic assays (Fig. 3A and Table 3). When tested by PRA, VFV



162 caused a dose-dependent inhibitory effect on HCMV replication (Fig. 3B), albeit with an  
163  $EC_{50}$  value higher than that of PCZ (13.3  $\mu$ M *versus* 3.3  $\mu$ M). However, given the highly  
164 hydrophobic nature of VFV (LogP 5.4), we reasoned that VFV could be poorly soluble in the  
165 semi-solid medium used for PRAs, thus virus yield reduction assays with VFV were also  
166 performed. VFV showed indeed a strong and dose-dependent inhibitory effect on infectious  
167 virus production with an  $EC_{50}$  in the low-micromolar range (Fig. 3C and Table 3), thus  
168 suggesting that hCYP51 enzymatic activity is required for the production of infectious  
169 HCMV progeny.

170 **HCMV infection activates hCYP51 expression.** Since we observed that hCYP51  
171 enzymatic activity is required for HCMV replication, next we investigated whether its  
172 expression could be modulated during viral infection. Indeed, under normal conditions (i.e.,  
173 cells grown in regular cholesterol-containing medium), the cholesterol biosynthetic pathway  
174 is not stimulated. To analyze the modulation of *hCYP51* promoter upon HCMV infection, we  
175 transfected permissive U-373 MG cells with a plasmid encoding a reporter gene under the  
176 control of *hCYP51* promoter and then infected them with HCMV. As shown in Fig. 4A,  
177 infection with HCMV activated *hCYP51* promoter by about 40-fold. This upregulation was  
178 not observed when the transfected cells were infected with a UV-inactivated virus, which is  
179 able to bind and enter into the cells but not to express viral genes, arguing that *de novo*  
180 synthesized HCMV proteins are required for *hCYP51* promoter activation (Fig. 4A). Upon  
181 infection of serum-fed HFFs, a ~2-fold increase in *hCYP51* mRNA level was observed  
182 during the early phase of HCMV replication (Fig. 4B) and an accumulation of hCYP51  
183 protein throughout the virus cycle was accordingly detected by both Western blot (Fig. 4C

184 and Fig. S1) and immunofluorescence analysis in living cells (Fig. S2). Altogether, these  
185 data support the view that HCMV infection activates *hCYP51* gene expression.

186 **Inhibition of hCYP51 enzyme during HCMV replication reduces the infectivity**

187 **of viral progeny.** As shown above, both VFV and PCZ inhibited hCYP51 enzyme, which

188 catalyzes the rate-limiting step in late cholesterologenesis. Thus, treatment of HCMV-infected

189 cells with these inhibitors should reduce *de novo* cholesterol synthesis during HCMV

190 replication. The intracellular presence of cholesterol during HCMV infection and within the

191 virus particle has been related to the infectivity of HCMV virions (31). We therefore

192 hypothesized that the virus particles produced in cells treated with hCYP51 inhibitors might

193 be less infective. To test this, we determined the particle-to-PFU ratio of cell-free HCMV

194 released from infected HFF cells treated with PCZ, VFV, or DMSO as a control, assuming

195 that each virus particle (infectious or defective) contained one genome. Treatment of

196 HCMV-infected cells for 120 h with either PCZ or VFV significantly reduced both the

197 number of HCMV genomes (Fig. 5A) and the infectivity of viral progeny, with a ~7-10-fold

198 increase in particle-to-PFU ratio with respect to DMSO-treated infected cells (Fig. 5B).

199 Altogether, the results from this section indicated that hCYP51 enzymatic activity is required

200 in the context of productive HCMV replication and could contribute to the generation of

201 infectious HCMV particles.

202 **GCV and PCZ act synergistically against HCMV replication in infected cells.**

203 We also tested by PRAs the antiviral efficacy of GCV in the absence or the presence of a

204 dose of PCZ (3  $\mu$ M) approximately equivalent to the mean concentration found in plasma by

205 therapeutic drug monitoring of patients treated with PCZ (25). Noteworthy, in the presence

206 of PCZ, GCV was ~~10~~ more potent against HCMV than in the presence of the vehicle

207 DMSO ( $EC_{50}$  0.13  $\mu$ M for the combination of GCV + PCZ *versus* 1.44  $\mu$ M for GCV +  
208 DMSO, Fig. 6). Considering these results, we investigated further the effects of the  
209 combination of PCZ with GCV against the replication of HCMV AD169 by PRAs. As  
210 reported in Table 4, the combination of GCV and PCZ resulted in antiviral synergism at all  
211 drug combinations tested, since the Combination Index (CI) values calculated by applying  
212 the Chou & Talalay method (32) resulted all  $<0.9$  (Table 4). We did not observe any evident  
213 cell cytotoxicity when the drugs were tested in combination, thus the synergistic effect on the  
214 reduction of viral plaques number was most likely the result of combining two drugs with  
215 different targets and mechanisms of action. Beside a reduction in the absolute number, we  
216 also observed a clear reduction in the size of the plaques when GCV was used in  
217 combination with PCZ. The calculated Dose Reduction Indexes are reported in Table S3.  
218 Although future trials will be required to validate this observation in a clinical setting, these  
219 results suggest that a combination of GCV and PCZ might represent a new therapeutic  
220 strategy for the management of HCMV infections.

221

222

## 223 DISCUSSION

224 Our study stems from the observation of an overrepresentation of drugs belonging to  
225 the class of azolic antifungals among the molecules selected in a previous drug repurposing  
226 screening aimed at identifying new anti-HCMV compounds (12). Starting from this  
227 observation, we report for the first time that posaconazole, a drug already approved and used  
228 in both adult and pediatric immunosuppressed patients for prevention and as salvage therapy  
229 of fungal infections, is a potent inhibitor of HCMV replication in the low micromolar range.

230 By characterizing the anti-HCMV profile of PCZ, we found that upon treatment of  
231 infected cells with PCZ and VFV, two inhibitors of hCYP51 enzyme, the infectivity of the  
232 released HCMV particles is significantly reduced. Azolic antifungals have shown a  
233 polypharmacological profile mainly by affecting host cholesterol homeostasis and trafficking  
234 (26-29), in addition to be active against several unrelated viruses (11,18-21). In human cells,  
235 PCZ is known to interfere with several pathways, such as cholesterol trafficking from  
236 lysosomes by inhibiting the Niemann-Pick C1 protein transporter (26) and from ER by  
237 inhibiting oxysterol-binding protein (OSBP) cholesterol-shuttling protein (11,19), as well as  
238 cholesterologenesis by inhibiting hCYP51 enzyme (29,30). The inhibition of OSBP, the target  
239 of PCZ against single-stranded positive RNA viruses, might be however likely excluded as a  
240 possible mechanism of anti-HCMV activity of PCZ on the basis of the anti-HCMV activity  
241 exhibited also by imidazolic antifungals such as miconazole and econazole (Table 1 and  
242 Table 2), which do not bind OSBP (12,19).

243 The reduced infectivity of HCMV particles released from PCZ-treated cells could be  
244 caused by the block of cholesterol trafficking and hCYP51 inhibition caused by the drug,  
245 leading to lower levels of available cholesterol. In fact, changes in the cholesterol uptake and  
246 efflux have been observed during HCMV infection (31,33), and it has been reported that  
247 virion cholesterol content is crucial for the infectivity of HCMV progeny (31). Later during  
248 infection, when appropriate levels of new cholesterol molecules could be required by HCMV  
249 to produce infectious virions, inhibition of hCYP51-mediated cholesterologenesis exerted by  
250 PCZ would thus have a detrimental effect on the infectivity of the released viral progeny.  
251 However, this hypothesis remains to be tested. Nonetheless, our data suggest a role of the  
252 late cholesterologenesis mediated by hCYP51 in the production of infectious HCMV virions.

253 Indeed, we found that HCMV induces hCYP51 expression during infection. Importantly, a  
254 functional role for hCYP51 in the context of productive HCMV infection was validated by a  
255 pharmacological approach with the enzymatic inhibitors VFV and PCZ. Furthermore, the  
256 observed decrease in virus yield and in infectivity of virus particles produced upon inhibition  
257 of hCYP51 enzymatic activity supports the view that new cholesterol molecules are indeed  
258 required for the generation of infectious virus particles. Accumulation of new cholesterol  
259 molecules might contribute in conferring appropriate membrane fluidity to the enlarged  
260 cytomegalic cell, in the organization of virion, or in the targeting and proper localization of  
261 viral proteins within the envelope or mature virus particle. Accordingly, in other virus  
262 models, both virion cholesterol content and effective cholesterologenesis have been reported to  
263 affect virus infectivity. Depletion of cholesterol during hepatitis B virus infection was shown  
264 to induce in fact a topologic change of the large envelope protein that renders the virus non-  
265 infectious (34). Interestingly, also HIV-1 Nef protein induces hCYP51 expression to increase  
266 *de novo* cholesterol synthesis and enhance virions infectivity (35), and low levels of  
267 cholesterologenesis and cholesterol uptake have been related to a lower HIV trans-infection  
268 ability and a slower disease progression *in vivo* (36).

269 Posaconazole peak levels (C<sub>max</sub>) in plasma of treated patients either under  
270 prophylaxis or treatment of invasive fungal infections depend on a series of different factors,  
271 among which there are the formulation, the posology, the diet, and of course the  
272 administered dose (37). During prophylaxis in patients at high risk of both fungal and  
273 HCMV infections and for therapeutic use in the treatment of mycosis, posaconazole peak  
274 levels are reported to be averagely 1.5-2 µg/ml, corresponding to 2.2-2.9 µM (41-42), and  
275 thus approximately equal to or below the EC<sub>50</sub>s that we found for posaconazole against

276 HCMV (Table 2). However, anti-HCMV activity for posaconazole within a clinically  
277 achievable range could be obtained either by synergistic combination with GCV or by  
278 developing a better formulation.

279 We also observed that PCZ exerted anti-HCMV activity against both wild-type  
280 clinical isolates and drug-resistant viral strains in a cell-type independent manner.  
281 Importantly, we demonstrated that GCV and PCZ act synergistically in inhibiting HCMV  
282 replication in infected cells with a combination effect ranging from moderate to very strong  
283 synergism. The analysis of our data with Calcsyn software allowed the determination of the  
284 simulated Dose Reduction Index (DRI) for both GCV and PCZ. DRI estimates the extent to  
285 which GCV levels may be reduced when used in synergistic combination with PCZ to  
286 achieve effect levels compared with GCV used alone (32). For example, 90% inhibition of  
287 HCMV replication may be potentially obtained by reducing by 10- and 6-fold GCV and  
288 PCZ, respectively (Table S3), a condition that could be clinically achievable in treated  
289 patients. However, this simulation remains to be clinically tested *in vivo*.

290 In conclusion, our study suggests that repurposing of posaconazole against HCMV  
291 both alone and in combination with GCV could foster the development of new antiviral  
292 strategies against this important viral pathogen, exploiting its dual antimicrobial activity, and  
293 contribute to understand how HCMV manipulates host pathways for a productive replication.

294

295

## 296 MATERIALS AND METHODS

297 **Compounds.** Ganciclovir (GCV), foscarnet (FOS), and all the antifungal drugs were  
298 from Sigma-Aldrich. Cidofovir (CDV, Vistide) was from Gilead Sciences. VFV ((*R*)-*N*-(1-

299 (3,4'-difluoro-[1,1'-biphenyl]-4-yl)-2-(1*H*-imidazol-1-yl)ethyl)-4-(5-phenyl-1,3,4-oxadiazol-  
300 2-yl)benzamide) was synthesized at Vanderbilt University as described (43). For cellular  
301 assays, 100× stock of VFV was prepared in 25% dimethylsulfoxide (DMSO)/34% aqueous  
302 2-hydroxypropyl- $\beta$ -cyclodextrin (v/v) purchased from Sigma.

303 **Cells and viruses.** Human Foreskin Fibroblast (HFF), ARPE-19, and U-373 MG were all  
304 from the American Type Culture Collection (ATCC) and were cultured in Dulbecco  
305 modified Eagle's medium (DMEM) (Life Technologies) supplemented with 10% fetal  
306 bovine serum (FBS, Life Technologies), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin  
307 sulfate (P/S, both from Life Technologies). Human dermal microvascular endothelial cells  
308 (HMVECs) (CC-2543) were obtained from Clonetics and cultured in endothelial growth  
309 medium (EGM) (Clonetics). All cell cultures were maintained at 37°C in a humidified  
310 atmosphere supplemented with 5% CO<sub>2</sub>.

311 HCMV (strain AD169) was purchased from the ATCC. HCMV TB40E-UL32-EGFP  
312 (kindly provided by C. Sinzger, University of Ulm, Germany) was previously described (44)  
313 as well as HCMV VR1814 (kindly provided by G. Gerna, IRCCS Policlinico San Matteo,  
314 Pavia, Italy) recovered from a cervical swab from a pregnant woman (45). HCMV 388438U  
315 clinical isolate was collected from a urine sample at the Microbiology and Virology Unity of  
316 Padua University Hospital (Italy) and was under passage 4 after primary isolation. HCMV  
317 strains resistant to antiviral drugs were obtained from the NIH AIDS Research and Reference  
318 Reagent Program (Rockville, MD) and previously described (46). HCMV TR was  
319 reconstituted by transfecting HFFs with the corresponding TR-BAC that was prepared from  
320 the TR clinical strain resistant to GCV and CDV and isolated from an ocular specimen (47).

321 Reconstitution of BAC-derived TR strain in fibroblasts generated infectious virus that  
322 retains the ability to infect both endothelial and epithelial cells (48).

323 **Plaque reduction assays.** Plaque reduction assays (PRAs) with HCMV were  
324 performed as previously described (49). Briefly, HFF, ARPE-19, and HMVEC cells were  
325 seeded at a density of  $1.5 \times 10^5$  cells per well in 24-well plates. The next day, the cells were  
326 infected at 37°C with 80 Plaque Forming Unit (PFU) per well of the different viruses in  
327 serum-free DMEM. At 2 h p.i., the inocula were removed, cells were washed, and media  
328 containing various concentrations of each compound, 2% FBS, and 0.6% methylcellulose  
329 were added. After 10 days of incubation at 37°C, cell monolayers were fixed and stained  
330 with crystal violet, and viral plaques were counted.

331 **Cytotoxicity assays.** The cytotoxicity of tested compounds was determined by the 3-  
332 (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) method  
333 as described previously (50).

334 **Virus yield reduction assays.** For virus yield reduction assays, HFF cells were  
335 plated at  $2 \times 10^4$  cells per well in 96-well plates, incubated overnight, and infected the next  
336 day with HCMV AD169 at MOI = 0.1 PFU/cell. After virus adsorption for 2 h at 37°C, cells  
337 were washed and incubated with 0.2 ml of fresh medium containing 5% FBS in the absence  
338 or in the presence of test compounds. Plates were incubated for 5 days at 37°C, and then  
339 subjected to one cycle of freezing and thawing. Titres were determined by transferring 0.1 ml  
340 aliquots from each well to a fresh 96-well monolayer culture of HFF cells, followed by 1:5  
341 serial dilutions across the plate. Cultures were incubated for 7 days and at the end of  
342 incubation they were fixed and stained, and the numbers of plaques were determined.



343       **Enzymatic assays *in vitro*.** Recombinant human CYP51 and its redox partner  
344       NADPH-cytochrome P450 reductase (CPR) were expressed in *Escherichia coli* and purified  
345       as described previously (30). The standard reaction mixture contained 0.5  $\mu$ M hCYP51 and  
346       1.0  $\mu$ M CPR), 100  $\mu$ M L- $\alpha$ -1,2-dilauroyl-*sng*lycerophosphocholine, 0.4 mg/ml isocitrate  
347       dehydrogenase, and 25 mM sodium isocitrate in 50 mM potassium phosphate buffer (pH 7.2)  
348       containing 10% glycerol (v/v). After addition of the radiolabeled ([3-<sup>3</sup>H]) lanosterol (~4,000  
349       dpm/nmol; dissolved in 45% HPCD, w/v, final concentration 50  $\mu$ M) and inhibitors  
350       (concentration range 0.1 - 20  $\mu$ M), the mixture was preincubated for 30 s at 37 °C in a  
351       shaking water bath, the reaction was initiated by the addition of 100  $\mu$ M NADPH and  
352       stopped by extraction of the sterols with 5 ml of ethyl acetate. The extracted sterols were  
353       dried, dissolved in methanol, and analyzed by a reversed-phase HPLC system (Waters)  
354       equipped with a  $\beta$ -RAM detector (INUS Systems) using a NovaPak octadecylsilane (C<sub>18</sub>)  
355       column and a linear gradient water/acetonitrile/methanol (1.0:4.5:4.5, v/v/v) (solvent A) to  
356       CH<sub>3</sub>OH (solvent B), increasing from 0 to 100% B for 30 min at a flow rate of 1.0 ml/min.  
357       The IC<sub>50</sub> values were calculated using GraphPad Prism 6, with the percentage of lanosterol  
358       converted being plotted against inhibitor concentration and the curves fitted with non-linear  
359       regression (log(inhibitor) vs. normalized response - variable slope).

360       **Plasmids.** The pCYP51-luc plasmid, which contains the -314/+343 human *CYP51*  
361       (*hCYP51*) gene promoter region upstream of the luciferase reporter gene was kindly  
362       provided by D. Rozman (Centre for Functional Genomics and Bio-Chips Institute of  
363       Biochemistry, Faculty of Medicine University of Ljubljana, Slovenia) and was previously  
364       described (51). pGAPDH-eGFP plasmid, which contains the promoter region of cellular

365 *GAPDH* gene upstream of the *enhanced Green Fluorescent Protein* (eGFP) gene was  
366 previously described (41) and used as a control of transfection efficiency.

367 **Cell transfections and HCMV infection.** For the transfection/infection experiments  
368 with HCMV, U-373 MG cells were grown on 24-well plates and co-transfected using  
369 calcium phosphate (Calcium Phosphate Transfection Kit, Sigma) with 1  $\mu$ g of pCYP51-luc  
370 plasmid along with 0.2  $\mu$ g of pGAPDH-eGFP plasmid as a control to normalize transfection  
371 efficiency. The next day, transfected cells were either mock-infected or infected with HCMV  
372 AD169 at MOI = 0.5 PFU/cell for 2 h and then incubated with 5% FBS-containing medium.  
373 At 48 h post-infection, luciferase activity as well as eGFP expression were measured. For all  
374 the experiments, the values were normalized by dividing the values obtained for luciferase  
375 (LU) by the fluorescence units (FU) obtained for eGFP expression and expressed as relative  
376 luciferase units (RLU). For UV-inactivation, a procedure previously described was followed  
377 (52). Briefly, HCMV diluted in serum-free DMEM was exposed for 8 min at a distance of 4  
378 cm under a UV-light (VL-6MC, 254 nm, 6w). Inactivation of the virus was assessed by  
379 immunofluorescence.

380 **Quantification of gene expression.** HFF cells were seeded in 6-well plates at  $6 \times 10^5$   
381 cells/well and incubated o/n at 37°C. In case of infection, the next day they were infected  
382 with HCMV AD169 at MOI = 1 PFU/cell for 2 h and then incubated with 5% FBS-  
383 containing medium. Total RNA was extracted from samples collected at different times p.i.  
384 using a total RNA Purification Plus Kit (Norgen Biotek) according to the manufacturer's  
385 protocol. cDNA was generated from RNA (2  $\mu$ g) using random primers (Applied  
386 Biosystems) and M-MLV reverse transcriptase (Applied Biosystems). qPCR was performed  
387 with SYBR green reagent (Applied Biosystems) according to the manufacturer's instructions

388 on a 7900 HT Fast Real-Time PCR System (Applied Biosystems) using primers for *hCYP51*,  
389 *GAPDH*, and HCMV *UL54* genes (sequences reported in Table S1). The relative changes in  
390 gene expression were calculated by means of the  $\Delta\Delta C_t$  method (53) using *GAPDH* to  
391 normalize data.

392 **Western Blot.** For the analysis by Western blot of the induction hCYP51 during  
393 HCMV infection, subconfluent HFF cells in 6-well plates were infected with HCMV AD169  
394 at a MOI = 0.5 PFU/cell. Whole-cell protein extracts were prepared at different times as  
395 previously described (12) and then analyzed by Western blot with different antibodies listed  
396 in Table S2. Immunocomplexes were detected with the appropriate secondary anti-  
397 immunoglobulin Abs conjugated to horseradish peroxidase (Life Technologies).  
398 Densitometry analysis was performed with ImageJ software (<https://imagej.nih.gov/ij/>).

399 **Immunofluorescence and confocal microscopy analysis.** For confocal laser-  
400 scanning microscopy analysis, HFFs were infected with HCMV AD169 at an MOI = 0.25  
401 PFU/cell. At different times p.i., cells were fixed with 4% paraformaldehyde in PBS 1× for  
402 15 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 20  
403 min at RT. After washing extensively with PBS, cells were incubated first with 4% FBS in  
404 PBS for 1 h at room temperature and then with different primary antibodies (listed in Table  
405 S2) diluted in FBS 4% in PBS 1× for 1 h at 37°C under shaking. Cells were then washed  
406 extensively with 4% FBS in PBS 1× and incubated with secondary fluorochrome-conjugated  
407 antibodies (listed in Table S2) for 1 h at 37 °C. Nuclei were stained by incubation for 20 min  
408 with Draq5 (1:8,000 in PBS 1×). Cells were imaged using a Nikon Eclipse Ti-E microscope.

409 **Particle-to-PFU ratio determination.** To determine the particle-to-PFU ratio of  
410 HCMV produced in the presence of test compounds, HFFs were seeded at  $2 \times 10^4$  cells per

well in 96-well plates, incubated overnight, and infected the next day with HCMV AD169 at MOI = 0.5 PFU/cell. After virus adsorption for 2 h at 37°C, cells were washed and incubated with 0.2 ml of fresh medium containing 5% FBS in the presence or in the absence of test compounds. Plates were incubated for 5 days at 37°C. At the end of the incubation, 0.05 ml of supernatants were used to determine the number of virus particles that were produced under the different experimental conditions, while 0.05 ml were titrated on fresh monolayers of HFF cells as previously described, to determine the number of PFUs present in the same volume of supernatant. For virus particles determination, 0.05 ml of supernatants were incubated with 0.2% SDS and proteinase K for 1 h at 56°C and then for 15 min at 95°C to inactivate proteinase K. Then, viral DNA was extracted with DNA purification kit (Promega) and quantified by qPCR as described below. The particle-to-PFU ratio was determined by dividing the number of HCMV genomes by the number of PFU determined in the same volume of supernatant derived from the same sample.

**Quantification of viral genomes.** To quantify the HCMV genomes in 0.05 ml of supernatants derived from the different samples collected at 120 h p.i., quantitative Real-Time PCR (qPCR) was performed as previously described (46). The number of viral genomes was normalized to the cellular *β-globin* gene copies. The sequences of the oligonucleotides used are listed in Table S1.

**Drug combination studies.** To evaluate the combined effects of PCZ and GCV on HCMV AD169 replication, plaque reduction assays were performed as described above using 0.25x, 0.50x, 1x, 2x, and 4x EC<sub>50</sub> for each combination of PCZ and GCV at equipotent ratio. The 2-drug combination effects were assessed using the Chou-Talalay method (32)

433 based on mass-action law based dynamic theory computed in the CalcuSyn software version  
434 2.0 (Biosoft, Cambridge, UK).

435 **Statistical analysis.** All statistical analyses were performed using GraphPad Prism  
436 version 6.0.

437  
438  
439

#### 440 SUPPLEMENTAL MATERIAL

441 Supplemental material to this article may be found at journal website.

442

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455

#### 456 DECLARATION OF INTERESTS

457

458 B.M., A. Luganini, G.G., and A. Loregian have filed a provisional patent on the use of  
459 posaconazole alone and in combination with ganciclovir for the treatment of pathological  
460 condition(s) associated with cytomegalovirus infection.

461

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- 632

633

## 634 **FIGURE LEGENDS**

635 **FIG 1 Susceptibility of HCMV to approved azolic antifungals.** Plaque reduction assays  
636 were performed in HFF infected with HCMV and treated with different doses (from 0.1 to 25  
637  $\mu$ M) of the indicated test compounds or GCV as a control. (A) Dose-dependent inhibition of  
638 HCMV AD169 replication by PCZ and KTZ. (B) Absence of significant inhibition of  
639 HCMV AD169 replication by FCZ, ITZ, and VCZ. (ITZ could be tested only up to 10  $\mu$ M  
640 due to solubility issues). Graph represents mean  $\pm$  SD of  $n \geq 3$  independent experiments in  
641 duplicate.

642 **FIG 2 Dose-dependent inhibition of the replication of indicated HCMV strains by**  
643 **posaconazole.** Plaque reduction assays were performed in HFF infected with the indicated  
644 HCMV strains and treated with different doses (from 0.1 to 25  $\mu$ M) of PCZ. Graphs  
645 represents mean  $\pm$  SD of n = 3 independent experiments in duplicate.

646 **FIG 3 Inhibition of host hCYP51 affects HCMV replication.** (A) Inhibition of enzymatic  
647 activity of purified hCYP51 by VFV, PCZ, and VCZ (2 min reaction). Graph represents the  
648 mean  $\pm$  SD of n = 3 independent experiments in duplicate. (B) Plaque reduction assays were  
649 performed in HFF infected with HCMV AD169 and treated with different doses (from 0.1 to  
650 25  $\mu$ M) of hCYP51 inhibitor VFV. Graphs represent the mean  $\pm$  SD of n  $\geq$  3 independent  
651 experiments in duplicate. (C) Dose-dependent inhibition by VFV of virus progeny  
652 production in HCMV-infected HFFs as determined by virus yield reduction assays. Graph  
653 represents the mean  $\pm$  SD of n = 4 independent experiments in duplicate.

654 **FIG 4 hCYP51 expression is activated during HCMV infection.** (A) Activation of  
655 *hCYP51* promoter in U-373 MG cells mock-infected or infected with either HCMV AD169  
656 or UV-inactivated HCMV. Reported data are expressed as Relative Luciferase Units  
657 (LU/FU, RLU), which are the luciferase units normalized to the fluorescence units derived  
658 from the expression of the co-transfected *eGFP* reporter gene. Graph represents the mean  $\pm$   
659 SD of n = 3 independent experiments in duplicate. Data were analyzed by a one-way  
660 ANOVA followed by Tukey's multiple comparison tests. \*\*\*p $\leq$ 0.0001; \*\*\*\*p<0.0001. (B)  
661 Analysis of *hCYP51* mRNA levels upon HCMV infection of HFF cells determined by qPCR  
662 at the indicated times. The mRNA levels of *UL54* were detected as a control for the  
663 progression of the infection at 24 and 48 h p.i. mRNA levels were normalized to cellular  
664 *GAPDH* and gene expression was reported as relative quantification (RQ) compared to

665 calibrator sample (mock-infected cells for *hCYP51* and HCMV-infected cells at 24 h p.i. for  
666 *UL54*). Graph represents the mean  $\pm$  SD of  $n = 3$  independent experiments in duplicate. (C)  
667 Analysis of hCYP51 and viral proteins expression during HCMV replication. Host hCYP51  
668 protein and viral IE antigens (IEA) were detected by Western blot both in mock-infected  
669 HFFs (M) and in HFFs infected with HCMV at an MOI = 0.5 PFU/cell at the indicated h p.i.  
670 Detection of host  $\beta$ -actin was used as a loading control. Molecular weights in kDalton are  
671 indicated on the left. Image of a representative experiment is showed.

672 **FIG 5 Effects of hCYP51 enzymatic activity inhibition on HCMV replication and**  
673 **infectivity.** (A) Pharmacological inhibition of hCYP51 reduces the number of HCMV  
674 genomes. HFF cells infected with HCMV at MOI = 0.5 PFU/cell were treated with 10  $\mu$ M  
675 PCZ or VFV, or 0.1% DMSO as a control for 120 h. HCMV genome copies in the  
676 supernatant of each sample were then determined by qPCR. Graph represents the mean  $\pm$  SD  
677 of  $n = 3$  independent experiments in quadruplicate. Data were analyzed by a one-way  
678 ANOVA followed by Dunnett's multiple comparison test. \*\*\* $p < 0.001$ ; \*\* $p < 0.005$  compared  
679 to control (DMSO-treated, infected sample). (B) Pharmacological inhibition of hCYP51  
680 reduces the infectivity of viral particles. Particle-to-PFU ratios were obtained by dividing the  
681 number of HCMV particles collected from supernatants derived from HFFs infected at MOI  
682 = 0.5 PFU/cell and treated with test compounds (determined by qPCR) by the viral titers  
683 obtained in the same sample volume (determined by titration on fresh monolayers). Graph  
684 represents the mean  $\pm$  SD of  $n = 3$  independent experiments in quadruplicate. Data were  
685 analyzed by a one-way ANOVA followed by Dunnett's multiple comparison tests. \* $p < 0.05$ ;  
686 \*\* $p < 0.005$ , compared to control (DMSO-treated, infected sample).

687 **FIG 6 Therapeutic dose of posaconazole enhances anti-HCMV activity of ganciclovir.**

688 Antiviral efficacy of GCV against HCMV AD169 in the absence (+ DMSO) or the presence  
689 (+ PCZ) of 3  $\mu$ M PCZ as determined by PRA. Graph represents the mean  $\pm$  SD of  $n \geq 3$   
690 independent experiments in duplicate.

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698**Table 1. Antiviral activity of antifungal drugs against HCMV AD169**

Compound (Abbreviation)	EC <sub>50</sub> <sup>b</sup> (μM)	CC <sub>50</sub> <sup>c</sup> (μM)	SI <sup>d</sup>
Clotrimazole (CTZ) <sup>a</sup>	0.8 ± 0.6	40 ± 28	50
Econazole (ECZ) <sup>a</sup>	5.7 ± 1.7	85 ± 13	17
Fluconazole (FCZ)	> 25	>250	>10
Itraconazole (ITZ)	>10	N.D.	N.D.
Ketoconazole (KTZ)	14.4 ± 4.6	116 ± 6	8
Miconazole (MCZ) <sup>a</sup>	3.2 ± 0.8	55 ± 16	17
Posaconazole (PCZ)	3.3 ± 0.5	>250	>76
Voriconazole (VCZ)	> 25	> 250	>10
Ganciclovir (GCV)	2.3 ± 0.6	>500	>217

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<sup>a</sup> Reported in Reference 12.

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<sup>b</sup> 50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs against HCMV AD169 in HFF cells. Reported values represent the means ± SD of data derived from n ≥ 3 independent experiments in duplicate. GCV was included as a positive control.

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<sup>c</sup> Compound concentration that produces 50% of cytotoxicity, as determined by MTT assays in HFF cells.

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Reported values represent the means ± SD of data derived from two independent experiments in duplicate.

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<sup>d</sup> SI, Selectivity Index (determined as the ratio between CC<sub>50</sub> and EC<sub>50</sub>).

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N.D., Not Determined.

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**Table 2. Antiviral activity of antifungal drugs against different HCMV strains in different cell types**

HCMV strain	Cell type	Antiviral activity EC <sub>50</sub> <sup>a</sup> (μM)			
		ECZ	MCZ	PCZ	Control <sup>b</sup>
TB40-UL32-EGFP	HFF	4.5 ± 0.7	3.8 ± 0.4	3.5 ± 1.8	3.5 ± 0.7
VR1814	HFF	3.9 ± 2.1	4.3 ± 1.1	3.5 ± 0.7	2.3 ± 1.3
388438U	HFF	N.D.	N.D.	3.2 ± 0.4	3.2 ± 0.8
759 <sup>f</sup> D100	HFF	4.3 ± 1.1	4.5 ± 1.8	2.6 ± 1.2	75 ± 12
GDG <sup>f</sup> P53	HFF	3.4 ± 0.9	2.6 ± 0.3	3.5 ± 1.1	1.9 ± 1.8
PFA <sup>f</sup> D100	HFF	4.2 ± 0.1	3.7 ± 0.6	4.8 ± 1.5	250 ± 18
TR	HFF	N.D.	N.D.	3.7 ± 1.3	N.D.
TR	ARPE-19	N.D.	N.D.	4.2 ± 1.8	N.D.
TR	HMVEC	N.D.	N.D.	4.8 ± 2.0	N.D.

<sup>a</sup> 50% Effective Concentration, the compound concentration that inhibits 50% of plaque formation, as determined by PRAs in HFFs, and for TR strain also in epithelial (ARPE-19) and endothelial (HMVECs) cells. Reported values represent the means ± SD of data derived from n ≥ 3 independent experiments in duplicate.

<sup>b</sup> GCV was used as a control for all strains except for PFA<sup>f</sup>D100, for which FOS was used.

N.D., Not Determined.

**Table 3. Activity of posaconazole and VFV in virus yield reduction assays and in enzymatic assays *in vitro***

Compound	Antiviral Activity ( $\mu\text{M}$ )		Inhibitory Activity ( $\mu\text{M}$ )
	$\text{EC}_{50}^{\text{a}}$	$\text{EC}_{90}^{\text{b}}$	$\text{IC}_{50}^{\text{c}}$
PCZ	$2.2 \pm 1.1$	$7.1 \pm 1.1$	$8.0 \pm 2.5$
VFV	$1.2 \pm 0.9$	$10.1 \pm 1.3$	$0.5 \pm 0.2$
VCZ	N.D.	N.D.	>100

<sup>a</sup> 50% Effective Concentration, the compound concentration that inhibits 50% of virus yield, as determined by titration in HFF cells. Reported values represent the means  $\pm$  SD of data derived from n = 4 independent experiments in duplicate.

<sup>b</sup> 90% Effective Concentration, the compound concentration that inhibits 90% of virus yield, as determined by titration in HFF cells. Reported values represent the means  $\pm$  SD of data derived from n = 4 independent experiments in duplicate.

<sup>c</sup> 50% Inhibitory Concentration, the compound concentration that causes a 50% decrease in the rate of lanosterol conversion, as determined by reconstitution of the hCYP51 activity *in vitro*, 2 min reaction. Reported values represent the means  $\pm$  SD of data derived from n = 3 independent experiments in duplicate and calculated using GraphPad Prism 6.0 (dose-response –inhibition).

N.D., Not Determined.

**Table 4. Analysis of the effects of the combination of GCV and PCZ against HCMV replication**

GCV/PCZ combination at equipotent ratio (fold of EC <sub>50</sub> <sup>a</sup> )	CI <sup>b</sup>	Drug Combination effect <sup>c</sup>
0.25	0.625 ± 0.169	Synergism
0.5	0.780 ± 0.031	Moderate synergism
1	0.353 ± 0.278	Synergism
2	0.075 ± 0.039	Very strong synergism
4	0.064 ± 0.042	Very strong synergism

<sup>a</sup> Fold of 50% Effective Concentration for GCV/PCZ yielding an equipotent concentration ratio between the two combined drugs. The EC<sub>50</sub> values was determined by PRAs against HCMV AD169 in HFF cells for each drug alone or in combination at concentrations starting from 4-fold to 0.25-fold the equipotent ratio of the drugs considering ratio 1:1.33, approximated values from Table 1.

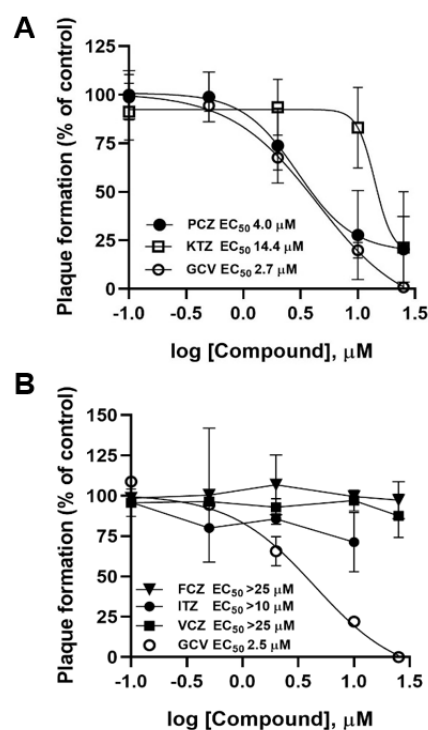
<sup>b</sup> Combination Index, obtained by computational analysis with Calcsyn software. Reported values represent means ± SD of data derived from n = 3 independent experiments in triplicate.

<sup>c</sup> Drug combination effect defined as: very strong synergism for CI<0.1; strong synergism for 0.1<CI<0.3; synergism for 0.3<CI<0.7; moderate synergism for 0.7<CI<0.9, according to (32).



814 **Figure 1**

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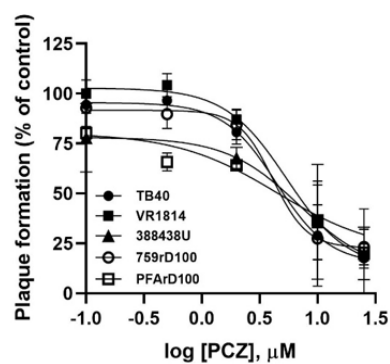
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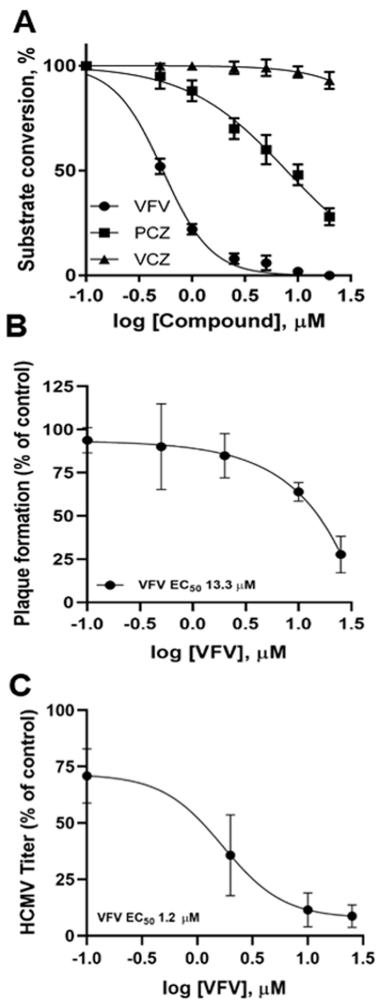
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Figure 2



843 **Figure 3**

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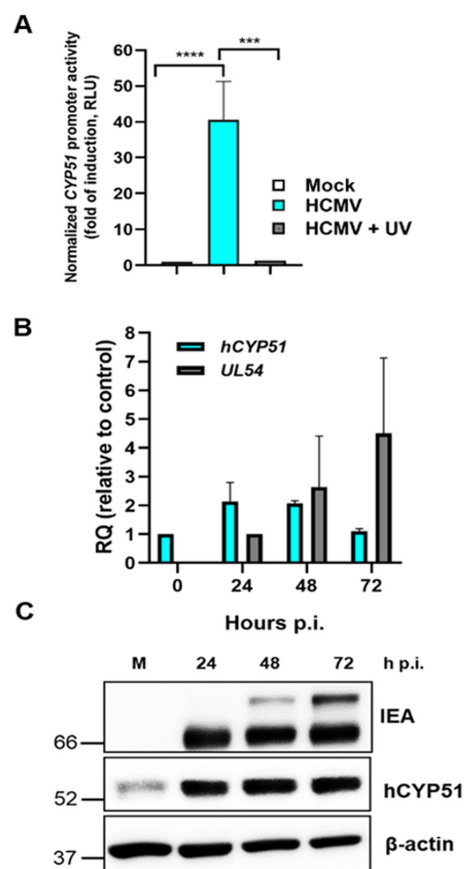
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850 **Figure 4**



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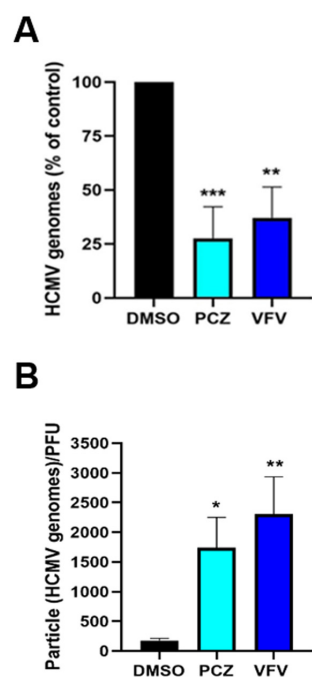
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861 **Figure 5**

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Figure 6

